— SECTION E —

MEDICAL SCIENCES

Contents

BLOOD AND SPINAL FLUID GUANIDINE IN EPILEPSY[1]

BY K. A. C. ELLIOTT AND NORA HENDERSON

Abstract

It has been reported that the amount of guanidine-like substances in the blood of "essential" epileptics is higher than the normal and increases considerably before and during seizures. In the present study no sign of a raised "guanidine" level was found in the blood or spinal fluid or any type of epileptic patient.

Murray and Hoffman (3) reported that in 15 cases of "essential epilepsy" the basal level of substances estimated as guanidine in the blood was higher than in normal subjects and that it increased during the aura, reached a considerably higher value during a convulsion, and was still somewhat above the basal level, one hour after the convulsion. The averages of all the figures given, in mgm. per 100 ml., were 0.41 basal, 0.70 aura, 1.23 in convulsion, and 0.54 one hour postconvulsion, but there was considerable individual variation.

Guanidine is a convulsant and some of its pharmacological effects resemble those of eserine. It is closely related to creatine and phosphocreatine, which are intimately concerned in the chemical dynamics of nervous and other tissues. The guanidine group is also part of the purine and pyrimidine nuclei, which constitute part of a number of important coenzymes as well as nucleic acid derivatives. The finding of an increase in guanidine-like substances in relation to epilepsy would therefore be an important clue to the nature of the epileptic process.

Palmer, Scott, and Elliott (4), who were interested in the possible relation of migraine to epilepsy, found no significant differences between the average blood "guanidine" levels of normal subjects and migraine patients (during or between attacks), epileptics (interictal), and various other patients. The value obtained was about the same as the basal value for epileptics given by Murray and Hoffmann. The latter authors gave no values themselves for normal subjects but accepted the value of 0.21 to 0.28 given by Andes and Myers (1). Palmer *et al.* concluded that the average basal value for epileptics was not different from normal but that the similar modifications of the analytical method used by themselves and by Murray and Hoffmann gave slightly higher values than the original method of Andes and Myers.

TABLE I

GUANIDINE-LIKE SUBSTANCES IN BLOOD AND SPINAL FLUID OF EPILEPTIC PATIENTS

Patient	Type, and time since, last seizure	"Guanidine" level mgm. per 100 ml.	
		Blood	Spinal fluid
R. L. Encephalopathy	General, 3.5 hr.	0.22	
M. W. Postencephalitis	General, 1 day	0.24	
M. B. Encephalopathy	In status epilepticus	0.51	
M. F. Neoplasm?	In status epilepticus	0.24	0.07
E. P. Birth injury	Continuous twitches*	0.25	0.10
J. H. " "	General, during seizure	0.42	
N. A. Focal epilepsy; cause unknown	General, 15 min.	0.25	0.10
C. C. Idiopathic epilepsy	General, 9 days	0.40	
J. T. " "	General, 6 days	0.47	
G. K. " "	General, 26 min., and petit mal every few min.	0.61	0.17
H. S. " "	General, 10 min.	0.39	
R. S. " "	General, 10 min.	0.37	
C. E. " "	General, 5 min.	0.50	
W. L. " "	General, during seizure	0.26	
J. M. " "	Petit mal,* 5 min.	0.19	
S. P. " "	Petit mal, 5 min.	0.35	
J. L. " "	Petit mal, 3 min.	0.36	
E. S. " "	Petit mal,* 1 min.	0.40	0.10
L. D. " "	Petit mal, 0 min.	0.26	
G. K. " "	Petit mal every few min.	0.59	
G. K. " "	Petit mal every few min.	0.43	0.15
Metrazol provoked seizures			
M. W. Postencephalitis	General, 4 min.	0.26	
Y. B. Idiopathic epilepsy	General, 0 min.	0.40	

* *Patient also had generalized seizures.*

In the present study, determinations have been made of substances estimated as guanidine in the blood and spinal fluid from a number of different types of patients showing epileptic symptoms.

The analytical method, a slight modification of the method of Andes and Myers (1), was the same as that used by Palmer *et al.* except that, in all but three cases (M.B., R.S., W.L.), deproteinization was achieved with trichloroacetic acid instead of tungstic acid. As soon as possible after withdrawal, one volume of blood or of spinal fluid was run into four volumes of 10% trichloroacetic acid and the analysis was carried out on 25 ml. of the filtrate. This was neutralized to about pH 7 with N sodium hydroxide, using small pieces of "Alkacid paper" to test the reaction, before carrying on the usual procedure. Tests showed that methyl guanidine added to the blood was about 70% recovered after either method of protein precipitation but that the basal values were about 0.14 mgm. % lower after trichloroacetic acid than after tungstic acid treatment. The results given here (except for the above-mentioned three) should be increased by about this amount to make them comparable with those of Palmer *et al.* and Murray and Hoffmann. Since

virtually the same method was used as previously (Palmer *et al.*) only three new determinations on normal blood were made; the figures obtained were 0.46, 0.29, 0.25.

The results obtained with blood and spinal fluid from epileptic patients are shown in Table I. In no case was a strikingly high value for blood "guanidine" obtained even when the blood was drawn during or very shortly after a seizure. The figures are generally within the normal range. Seizures induced in two epileptic patients by the slow administration of metrazol (2) did not give rise to high "guanidine" levels. In a few cases determinations were made on spinal fluid, drawn at the same time as the blood. The values obtained were low in all cases, and near the limit of sensitivity of the method.

There is thus no suggestion from this series of patients that the blood or spinal fluid "guanidine" level is affected in epileptic conditions. No explanation can be offered for the difference between these results and those of Murray and Hoffmann.

Acknowledgment

References

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4. PALMER, H. D., McNAIR SCOTT, D. B., and ELLIOTT, K. A. C. J. Lab. Clin. Med. 28 : 735. 1943.

ON THE ABO GENE AND Rh CHROMOSOME DISTRIBUTION IN THE WHITE POPULATION OF MANITOBA[1]

By B. Chown, R. F. Peterson, Marion Lewis, and Ann Hall

Abstract

In the Province of Manitoba, Canada, all men and women prior to marriage have their ABO and Rh blood groups determined. An analysis has been made of ABO and Rh data from blood samples of 3100 individuals of white race.

The calculated O, A, B gene frequencies in per cent based on the sample of 3100 individuals were as follows: O, 65.77; A, 26.07; B, 8.48. The calculated frequencies of Rh chromosomes for the same sample in per cent were as follows: R_1, 43.48; r, 39.55; R_2, 12.87; R_0, 1.91; R′, 1.24; R″, 0.73; R_z, 0.22; R_y, 0.00.

Frequencies of O, A, B genes and Rh chromosomes within the major national groups were also estimated and shown in comparison with the corresponding O, A, B gene frequencies for Great Britain and Rh chromosome frequencies for England.

The major national groups were compared in pairs by means of the Test of Homogeneity, with respect to the distribution, first of the ABO phenotypes, and then of the major Rh phenotypes. In general the Hebrew, Ukrainian, and Polish differed significantly from the remaining groups in distribution of the ABO phenotypes, due to a comparatively high frequency of the B phenotype in the former. With respect to the distribution of the Rh phenotypes the Hebrew group differed significantly from all others, due to a comparatively low incidence of the r phenotype.

Introduction

The previous studies in Manitoba (2, 3) were concerned with the Rh types in Canadians of Japanese race who had come to Manitoba and Western Ontario from British Columbia in 1942.

The purpose of the present paper is to study the distribution of the ABO genes and the Rh chromosomes in the white population of Manitoba through an analysis of data on 3100 individuals.

Material

A. *Blood Samples*

In the Province of Manitoba, Canada, every man and woman prior to marriage must, by law, have a serological test for evidence of syphilis done in the central laboratory of the Provincial Department of Health. The specimens of blood for this purpose are collected in sterile vacuum tubes, and after arrival at the laboratory are kept refrigerated. When that laboratory has finished with the blood, it is turned over to us. The specimens are, therefore, from two to seven days old when we receive them, but as they have been handled with care, and have been kept refrigerated, they reach us in good condition.

B. Sera

    (1) Anti-A and anti-B.*   These were prepared from donors with high
       titer, high avidity sera.   Their antibody titer was not increased by
       artificial means.

    (2) Anti-Rh.   The following sera were used:

        Anti-C. A single serum of our own, giving a strong reaction with
              saline suspended cells and having a titer of 1 : 32.
              Whether this serum is pure anti-C or is anti-C $+$ C^w
              is not known.   It contains an anti-D that agglutinates
              D-positive cells in albumen suspension but not in saline.

        Anti-D. All bloods were set up against three strong anti-D sera.
              All those that were negative to these three and positive
              to either anti-C or anti-E or both were checked with
              two further anti-D sera.   Undoubtedly even with this,
              some bloods that contained variants of Stratton's D^u
              antigen (7) were classed as D-negative since, as Race,
              Sanger, and Lawler (6) have reported, the elimination of
              all D^u bloods from apparent D-negatives requires
              "titrations in saline and in albumen against numerous
              anti-D sera, and tests by the anti-globulin and by the
              elution techniques".   However, the error is probably
              small, less than 1% (6).

        Anti-E. A single serum was used.**

        Anti-c. A single serum was used.**

Methods

    Agglutinogens A and B and agglutinins anti-A and anti-B were demonstrated
by the open-well slide technique, both agglutinogen and agglutinin content
of all bloods being determined.   The Rh agglutinogens were demonstrated by
our capillary method (1).

Results

    Table I is presented in order to indicate the proportions of the various
"races" or national groups making up the population of Manitoba.   The data
are from the 1941 Census as given in the Canada Year Book for 1945, to which
we have added a column showing the percentage of each "race".

    The data on which the present study is based are shown in Tables II and III.
The number of individuals of each ABO and Rh phenotype are shown for each
reported "racial origin" group.   The latter term requires explanation.

    In the official notice of marriage in the Province, from which notice our data
on "racial origin" are drawn, brides and grooms are required to state their

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    * *The blood groups specific substances A and B were kindly provided by Sharpe and Dohme
of Philadelphia.*

    ** *These sera were generously supplied by Dr. Louis K. Diamond, Boston.*

TABLE I

RACIAL ORIGINS OF THE POPULATION OF MANITOBA, 1941
(CANADA YEAR BOOK, 1945)

Racial origin	Number of individuals	Percentage
British Isles Races	360,560	49.41
English	168,917	23.15
Irish	76,156	10.44
Scottish	109,619	15.02
Other	5868	0.80
Other European Races	342,393	46.92
French	52,996	7.26
Austrian	4719	0.65
Belgian	6715	0.92
Bulgarian	24	0.003
Czech and Slovak	3702	0.51
Danish	3164	0.43
Finnish	808	0.11
German	41,479	5.68
Greek	399	0.05
Hungarian	2418	0.33
Icelandic	13,954	1.91
Italian	2482	0.34
Jewish	18,879	2.59
Lithuanian	407	0.06
Netherlands	39,204	5.37
Norwegian	5955	0.82
Polish	36,550	5.01
Rumanian	1438	0.20
Russian	6571	0.90
Swedish	9547	1.31
Ukrainian	89,762	12.30
Yugoslavic	451	0.06
Other	769	0.11
Asiatic Races	1788	0.24
Chinese	1248	0.17
Japanese	42	0.006
Other	498	0.07
Eskimo	1	0.0001
Indian	15,473	2.12
Negro	453	0.06
Other	8796	1.20
Not stated	280	0.04
	729,744	

"racial origin". No explanation of this term is given in the form itself, but as will be evident from Tables II and III, the term "race" is used mainly in a national sense. The term "Canadian" is not accepted in these reports, each individual being required to trace his ancestry to some other nationality or race. This is customarily done through the male line. Thus, for example, if an individual's paternal grandfather were English and the other three

TABLE II

DISTRIBUTION OF 3100 INDIVIDUALS OF WHITE RACE WITH RESPECT TO ABO PHENOTYPES

"Racial origin"	ABO phenotypes				Total
	O	A	B	AB	
American	1	1			2
Armenian		1			1
Austrian	5	13	2	1	21
Belgian	15	20			35
Czech	3	6	2	1	12
Danish	6	7		1	14
Dutch	52	49	6	6	113
English	372	331	71	18	792
Estonian		1			1
Finnish	2	1	1		4
Flemish			1		1
French	117	119	19	8	263
German	73	63	12	7	155
Greek	2	1			3
Hebrew	48	62	24	6	140
Hungarian	2	2		1	5
Icelandic	33	23	7	1	64
Irish	147	119	39	11	316
Italian	4	5	2	2	13
Latvian	2	1			3
Lithuanian			1		1
Norwegian	12	10	3	3	28
Polish	40	47	21	10	118
Rumanian	4	1		1	6
Russian	10	15	6	1	32
Ruthenian	2				2
Scottish	244	188	55	15	502
Spanish	1	1			2
Swiss	2	2			4
Swedish	21	25	7	3	56
Syrian	2		1	1	4
Ukrainian	108	141	82	19	350
Welsh	9	17	5	1	32
Yugoslav	2	2	1		5
Totals	1341	1274	368	117	3100

grandparents were Scotch, French, and German, respectively, the individual in question would be expected to report his "racial origin" as being English. This is also the procedure followed in making census returns.

We considered that it would be of interest not only to analyze the data from the sample of 3100 individuals as a whole but also to deal with some of the larger "racial" groups separately. We would caution the reader to keep in mind, however, the indefinite and rather artificial basis of the determination of these groups.

In order to have some measure of the extent to which our sample is representative of the Manitoba white population as a whole, we have attempted to compare the data on racial origins obtained from the marriage records of the 3100 individuals with those of the 1941 census as shown in Table I. However, the census figures do not show the proportion of whites in the second last

TABLE III

DISTRIBUTION OF 3100 INDIVIDUALS OF WHITE RACE WITH RESPECT TO Rh PHENOTYPE

"Racial origin"	R₁r (+ + − +)	R₁ (+ + − −)	r (− − − +)	R₂ (− + + −+)	R₁R₂ (+ + + +)	R₀ (− + − +)	R'r (+ − − +)	R'' (− − + +)	R₂ (+ + + −)	R' (+ − − −)	R'R'' (+ − + +)	Total
American	2											2
Armenian	1											1
Austrian	10	4	2	1	3	1						21
Belgian	9	6	9	6	5							35
Czech	4	3	4		1							12
Danish	5	3	2	2	2							14
Dutch	41	23	17	13	17	2						113
English	265	140	134	117	107	13	6	7	1	2		792
Estonian				1								1
Finnish	1	2		1								4
Flemish					1							1
French	79	52	36	38	49	3	2	3	1			263
German	43	29	26	25	26	1	2	2	1		1	155
Greek	3											3
Hebrew	49	41	11	7	24		2	1				140
Hungarian	1	1	1	3		1						5
Icelandic	15	17	6	11	12	1	1	1				64
Irish	108	60	56	46	39	3	3	1	1			316
Italian	5	4		1	2							13
Latvian	1	2										3
Lithuanian	1											1
Norwegian	6	9	1	5	7		1					28
Polish	33	30	16	16	21	1						118
Rumanian	2	2		4	2	1						6
Russian	12	5	4	4	6							32
Ruthenian	1				1							2
Scottish	171	86	81	85	66	6	2	3	2			502
Spanish	2	1		2	1	2						4
Swedish	12	13	9	10	8	1	1					56
Syrian	1	1	1			6	7	1				4
Ukrainian	130	57	65	47	37	2	7		1			350
Welsh	13	2	2	6	6		1					32
Yugoslav	2		2	1								5
Total	**1028**	**592**	**485**	**448**	**443**	**18**	**28**	**18**	**6**	**3**	**1**	**3100**

NOTE: *We assumed the absence of chromosome Ry in our population, since its existence had not been proved at the time this was written. Proof of its existence has since been established by Van den Bosch (Nature, 162 : 781-783, 1948). Since its frequency is probably less than 0.005 its omission does not materially affect our calculations.*

group of Table I designated as "Other", nor in the Asiatic races designated as "Other", and there is an unidentified group of 280 persons at the bottom of the table.   For these reasons we have compiled Table IV to include peoples

TABLE IV

NUMBER OF WHITE INDIVIDUALS OF EUROPEAN ORIGIN IN MANITOBA

"Racial origin"	1941 Census		Sample used in present study	
	Number	Percentage	Number	Percentage
English	168,917	24.03	792	25.59
Irish	76,156	10.83	316	10.21
Scottish	109,619	15.59	502	16.22
Other British Isles Races	5868	0.83	32	1.03
French	52,996	7.54	263	8.50
Austrian	4719	0.67	21	0.68
Belgian	6715	0.96	35	1.13
Czech and Slovak	3702	0.53	12	0.39
Danish	3164	0.45	14	0.45
Finnish	808	0.11	4	0.13
German	41,479	5.90	155	5.01
Greek	399	0.06	3	0.10
Hungarian	2418	0.34	5	0.16
Icelandic	13,954	1.98	64	2.07
Italian	2482	0.35	13	0.42
Jewish	18,879	2.69	140	4.52
Lithuanian	407	0.06	1	0.03
Netherlands	39,204	5.58	113	3.65
Norwegian	5955	0.85	28	0.90
Polish	36,550	5.20	118	3.81
Rumanian	1438	0.20	6	0.19
Russian	6571	0.93	32	1.03
Swedish	9547	1.36	56	1.81
Ukrainian	89,762	12.77	350	11.31
Yugoslavic	451	0.06	5	0.16
Other European Races	793	0.11	15	0.48
Total European	702,953		3095	

of European origin only.   This excludes the four Syrians and the one Armenian from our sample.   The Hebrews of Manitoba are of European ancestry, although the original source of this race is Asia.

The corresponding percentages shown in Table IV for the 1941 census and for our sample are strikingly similar, but when the Test of Homogeneity as described by Fisher (4) is applied to the data of Table IV (using the absolute numbers, not percentages) a χ^2 value of 143.779 is obtained with 25 degrees of freedom, indicating a statistically significant difference between our sample and the 1941 population with respect to the distribution of individuals in the designated racial groups.

The difference between the two series appears to be small from a biologic standpoint, but statistically significant.   When large numbers, such as we have here, are available for analysis, it is possible to demonstrate the statistical

significance of quite small differences. Such a difference as has been demonstrated here is not surprising since the two sets of data were taken at two different times and one set was obtained from a restricted age group.

Estimation of Gene Frequencies

The following formulae were used for calculating ABO gene frequencies:

$$O = \sqrt{(O)}$$

$$A = \sqrt{(O) + (A)} - \sqrt{(O)}$$

$$B = \sqrt{(O) + (B)} - \sqrt{(O)}$$

where O, A, and B represent gene frequencies and (O), (A), (B) represent phenotype frequencies.

For the calculation of frequencies of Rh chromosomes we have used the formulae provided by Race, Mourant, and McFarlane (5).

These are as follows:—

$$r = \sqrt{(---+)}$$

$$R' = \sqrt{(---+) + (+--+) + (+---)} - \sqrt{(---+)}$$

$$R'' = \sqrt{(---+) + (--++)} - \sqrt{(---+)}$$

$$R_0 = \sqrt{(---+) + (-+-+)} - \sqrt{(---+)}$$

$$R_1 = \sqrt{(++--) + (+--+)} - R'$$

$$R_2 = \sqrt{(++--) + (+--+) + (+++-)} - (R_1 + R')$$

$$R_z = 1 - (r + R' + R'' + R_0 + R_1 + R_z)$$

where the frequency of phenotype $---+$ (see Table III) is represented by $(---+)$.

The calculations were made on the entire sample of 3100 individuals and also on separate national groups having 100 or more individuals in the sample. The estimates of frequencies of OAB genes are presented in Table V, and the Rh chromosome frequencies in Table VI.

The most striking feature of Table V is the higher incidence of the gene B in Ukrainian, Polish, and Hebrew groups as compared to the other groups. Our estimates of O, A, and B gene frequencies based on the combined English, Scottish, and Welsh groups agree very closely with corresponding estimates for Great Britain made by Dobson and Ikin, and based on members of the Royal Air Force. The latter set of data was considered to be representative of Great Britain as a whole.

TABLE V

ESTIMATED FREQUENCIES OF THE GENES O, A, AND B IN MANITOBA WHITES IN COMPARISON
WITH DATA FROM GREAT BRITAIN

"Racial origin"	Number in sample	Frequency, %		
		O	A	B
English	792	68.53	25.68	6.25
Scottish	502	69.72	23.05	7.46
Irish	316	68.20	23.54	8.52
German	155	68.63	25.04	5.43
Dutch	113	67.84	26.70	3.81
French	263	66.70	28.03	5.21
Ukrainian	350	55.55	28.80	18.13
Polish	118	58.22	27.64	13.68
Hebrew	140	58.55	30.09	13.16
Others	351	63.16	28.52	8.26
Total	3100	65.77	26.07	8.48
Present study: English, Scottish, and Welsh	1326	68.65	24.92	6.85
Data from Great Britain*	190,177	68.31	25.69	6.00

* Aileen M. Dobson and Elizabeth W. Ikin. The ABO blood groups in the United Kingdom.
J. Path. Bact. 58 : 221-227. 1946.

In Table VI the Rh chromosomes appear to have fairly comparable distributions in the various groups except for the Hebrew group, in which there is a comparatively low incidence of r and high incidence of R_1 and R_0.

TABLE VI

ESTIMATED FREQUENCIES OF THE Rh CHROMOSOMES IN MANITOBA WHITES IN COMPARISON
WITH DATA FROM ENGLAND

"Racial origin"	Number in sample	Frequency, %						
		R_1	r	R_3	R_0	R'	R''	R_z
English	792	41.72	41.13	12.78	1.95	1.21	1.06	0.15
Scottish	502	41.38	40.17	15.29	1.46	0.49	0.74	0.47
Irish	316	43.54	42.10	11.77	1.11	1.11	0.37	0.00
German	154	42.56	41.09	11.71	0.78	2.31	1.55	0.00
Dutch	113	45.11	38.79	13.88	2.22	0.00	0.00	0.00
French	263	44.30	37.00	14.67	1.51	1.01	1.51	0.00
Ukrainian	350	40.50	43.09	11.87	1.94	2.26	0.00	0.33
Polish	118	50.12	36.82	10.79	1.13	1.13	0.00	0.00
Hebrew	140	52.98	28.03	10.13	5.78	2.44	0.00	0.64
Others	351	45.64	35.00	13.94	3.12	1.20	0.80	0.30
Total	3100	43.48	39.55	12.87	1.91	1.24	0.73	0.22
Data from England*	2000	42.04795	38.86134	14.10870	2.56677	0.98349	1.18819	0.24356

* R. R. Race, A. E. Mourant, Sylvia D. Lawlor, and Ruth Sanger. The Rh Chromosome
Frequencies in England. Blood, J. Hemat. 3 : 689-695. 1948.

Tests of Homogeneity

The homogeneity of different racial groups with respect to frequencies of O, A, and B genes may be tested by comparing two groups at a time using the Test of Homogeneity as described by Fisher (4). The same method can, of course, be applied in the case of Rh chromosomes. In both cases it is considered best to deal with the actual frequencies of phenotypes rather than the estimated frequencies of genes.

Such comparisons have been made, and typical analyses are shown in Tables VII and VIII. For some comparisons it was necessary, because of

TABLE VII

TEST OF HOMOGENEITY OF ENGLISH AND SCOTTISH GROUPS WITH RESPECT TO ABO PHENOTYPES

Phenotype	English	Scottish	Total
O	372	244	616
A	331	188	519
B	71	55	126
AB	18	15	33
Total	792	502	1294

$\chi^2 = 3.485.$ $n = 3.$ $P = .95$ *approx.*

TABLE VIII

TEST OF HOMOGENEITY OF ENGLISH AND SCOTTISH GROUPS WITH RESPECT TO Rh PHENOTYPES

Phenotype	English	Scottish	Total
r	134	81	215
R_1	140	86	226
R_1r	265	171	436
R_2	117	85	202
R_1R_2	107	66	173
Others	29	13	42
Total	792	502	1294

$\chi^2 = 2.235.$ $n = 5.$ $P = .80$ *approx.*

small numbers, to pool the data for the less frequent Rh phenotypes R_z, $R'r$, R', R'', R_1R'', and R_0 and we have, therefore, carried this out in all comparisons, using the six phenotypic classes shown in Table VIII. The tests as we have carried them out cannot yield any information regarding homogeneity with respect to the less frequent Rh phenotypes mentioned above, but only with respect to the major phenotypes.

The χ^2 values for tests of homogeneity of pairs of racial groups with respect to the proportion of individuals in the four phenotypes, A, B, O, and AB are shown in Table IX. The Hebrew, Ukrainian, and Polish groups do not differ

TABLE IX

χ^2 VALUES FOR TESTS OF HOMOGENEITY OF PAIRS OF RACIAL GROUPS WITH RESPECT TO THE PROPORTION OF INDIVIDUALS IN THE FOUR PHENOTYPES A, B, O, AND AB*

	English	Scottish	Irish	Dutch	German	French	Polish	Ukrainian	Hebrew
Hebrew	14.10	10.22	6.26	9.71	8.51	11.17	2.13	2.84	—
Ukrainian	60.49	39.18	23.57	20.64	22.28	34.34	2.86	—	
Polish	24.73	15.43	11.82	10.84	10.12	16.52	—		
French	1.99	5.72	5.98	1.66	1.25	—			
German	2.73	2.40	2.57	1.50	—				
Dutch	5.07	5.39	5.32	—					
Irish	4.82	0.65	—						
Scottish	3.48	—							
English	—								

* *The χ^2 value when $n = 3$ and $P = .05$ is 7.815.*

significantly among themselves but they differ significantly from all other groups in the relative proportions of the four phenotypes, apart from the Hebrew vs. Irish comparison, which gives a χ^2 value close to that for the 5% level of significance. The French, German, Dutch, Irish, Scotch, and English groups do not differ statistically among themselves.

Table X gives the χ^2 values for tests of homogeneity of pairs of racial groups with respect to the proportion of individuals in six classes based on Rh phenotypes. Here the Hebrew racial group differed significantly from all other

TABLE X

χ^2 VALUES FOR TESTS OF HOMOGENEITY OF PAIRS OF RACIAL GROUPS WITH RESPECT TO THE PROPORTION OF INDIVIDUALS IN SIX Rh PHENOTYPIC CLASSES*

	English	Scottish	Irish	Dutch	German	French	Polish	Ukrainian	Hebrew
Hebrew	25.54	28.83	23.94	10.90	18.59	15.51	11.28	26.55	—
Ukrainian	3.84	5.42	3.45	1.71	4.44	12.13	12.47	—	
Polish	7.56	7.45	5.66	7.88	3.27	2.25	—		
French	6.01	6.73	6.77	2.90	1.28	—			
German	5.21	3.48	4.08	3.81	—				
Dutch	2.77	2.95	1.65	—					
Irish	2.03	1.56	—						
Scottish	2.24	—							
English	—								

* *The classes used were r, R_1, R_1r, R_2, R_1R_2, and a class including the remaining phenotypes, namely R_z, $R'r$, R', R'', R_1R'', and R_0.*
The χ^2 value when $n = 5$ and $P = .05$ is 11.070.

groups except the Dutch, but in this comparison the χ^2 value is close to the 5% point of significance. The remaining eight national groups furnish 28 comparisons and only two of these (Ukrainian vs. French, and Ukrainian vs. Polish) show significant differences. It should be kept in mind that even if no real differences existed in these 28 comparisons a χ^2 value large enough to indicate significance would be expected to occur by chance once in 20 comparisons. It is possible that the two largest χ^2 values in the 28 referred to above are such chance values. Such consistently large χ^2 values as occurred for the comparisons of Hebrew with other groups, however, are so unlikely to have occurred by chance that it is safe to conclude that there is a real difference between the Hebrew and other white groups in frequencies of Rh phenotypes. It follows, of course, that there is also a difference in frequencies of Rh chromosomes.

It is possible, and indeed probable, that in some of the comparisons of national groups where differences with respect to frequencies of ABO or Rh phenotypes were not statistically significant, real differences actually existed, and that more extensive data will be needed to demonstrate such differences. A nonsignificant result in such comparisons as ours indicates that either no biologic difference existed with respect to the genes studied or else that the differences were not great enough to be demonstrable with the data at hand.

Finally, an interesting test of homogeneity is shown in Table XI in which our sample of 792 individuals of English origin in Manitoba is compared with the sample of 2000 individuals in England with respect to Rh phenotypes.

TABLE XI

TEST OF HOMOGENEITY OF 2000 INDIVIDUALS IN ENGLAND AND 792 INDIVIDUALS OF ENGLISH
DESCENT IN MANITOBA WITH RESPECT TO Rh PHENOTYPES

Reactions to antisera C, D, E, c	Phenotype	English (Race et al.) (a)	English in Manitoba (a')	Total $(a + a')$	$\dfrac{(an' - a'n)^2}{a + a'}$
+ + − −	R_1	373	140	513	463,261
+ + − +	$R_1 r$	689	265	954	257,980
+ + + +	$R_1 R_2$	270	107	377	68
− + + +	R_2	279	117	396	428,871
− − − +	r	307	134	441	1,400,954
+ − − +	$R' r$	16	6	22	20,527
− − + +	R''	19	7	26	42,242
− + − +	R_0	42	13	55	959,376
+ + + −	R_z	5	1	6	640,267
+ − − −	R'	0	2	2	8,000,000
		2000 (n)	792 (n')	2792	12,213,546

$$\chi^2 = \frac{12,213,546}{2000 \times 792} = 7.711. \qquad n = 9. \quad P = .5.$$

The P value of 0.5 indicates that the two series do not differ significantly in distribution of Rh phenotypes. If we pool the data for R_2 and R' to avoid the zero frequency in R' the P value becomes 0.95 with eight degrees of freedom, and the same conclusion is reached. The homogeneity is noteworthy in view of the data having been collected in different countries and the Rh reactions determined by different methods.

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ON ERGOTHIONEINE: ITS PREPARATION FROM BLOOD AND ERGOT[1]

By G. Hunter, G. D. Molnar, and N. J. Wight

Abstract

Ergothioneine has been prepared from pig blood and from ergot of rye. A poorer yield of ergothioneine was obtained from pig blood received in the Edmonton area than previously by Hunter and Eagles in the Toronto area. By a simplified method a yield of 0.26% has been obtained from ergot.

Introduction

The recent observations of Lawson and Rimington (8) on the action of ergothioneine on the thyroid gland of the rat has revived interest in this substance discovered in ergot of rye by Tanret (10) in 1909. In 1925 it was isolated from pig blood by Hunter and Eagles (5, 6) and later by Benedict, Newton, and Behre (1). The product from blood was identified as ergothioneine by Eagles and Johnson (2). It appeared probable that a supply of ergothioneine would be required for physiological and clinical studies, and this paper concerns significant observations on its preparation from pig blood and from ergot of rye.

Experimental

Preparation from Pig Blood

The methods used were essentially those already reported by Hunter and Eagles (5, 6), but our experience proved less fortunate. A routine yield obtained from about 3-gal. lots of pig blood in Toronto was about 0.7 gm. The best yield obtained in Edmonton from 10 lots of about 3 gal. of blood was 0.39 gm. and from the entire 30 gal. of blood used less than 2 gm. ergothioneine was obtained.

Preparation from Ergot

After some preliminary testing the following method was adopted.

Five hundred gm. of ergot corns was ground finely in a coffee mill. This was suspended in 2 liters water with the addition of 1 ml. glacial acetic acid. The mixture was brought to boil, with stirring at frequent intervals. While hot it was poured through cheesecloth and the residue was extracted in a filter press. The residue was washed twice, by bringing to boil with 1 liter water on each occasion, filtering, and expressing as above. The volume of filtrate and washings was 2660 ml.

The filtrate was now treated with a saturated aqueous solution of uranium acetate in very slight excess as tested, on a tile, by a 5% aqueous solution of

potassium ferrocyanide. The requisite amount of uranium acetate solution for the whole filtrate was determined on a 10 ml. aliquot. This was found to be 532 ml. It was added to the filtrate, and the mixture was well shaken. It was left to settle overnight, then the clear yellow supernatant liquid was siphoned off. The remainder was transferred to 250-ml. centrifuge bottles and further supernatant fluid collected.

The bulky precipitate in each bottle was thoroughly suspended in its own volume of water, recentrifuged, and the supernatant washings were added to the main filtrate. The washing was repeated twice. Total volume of filtrate and washings was 4450 ml.

The solution was now made 0.5 N acid with sulphuric acid and heated to 60° C. An aqueous suspension of cuprous oxide was added and the solution vigorously shaken. The cuprous oxide was added in slight excess as judged by a reddish tinge in the precipitate after shaking and maintaining at 60° C. for at least 30 min. The precipitate was allowed to settle. The supernatant liquid was siphoned off, and the remainder centrifuged. The copper precipitate was washed at 60° C. with its own volume of 0.5 N sulphuric acid, which had been brought to boil with a little cuprous oxide added. The washing was repeated twice.

The washed copper precipitate, containing some excess cuprous oxide, was suspended in hot water and freed from copper with hydrogen sulphide.

The filtrate and washings were freed from hydrogen sulphide, then brought to neutral to litmus paper by addition of warm barium hydroxide solution. At this point the solution was free from Ba and SO_4 ions.

The almost water clear filtrate was reduced *in vacuo* to about 15 ml. It was then boiled with a little charcoal, and the filtrate transferred to a boiling tube. The volume was reduced *in vacuo* to about 5 ml., when massive crystallization set in. The vacuum was released, and the contents of the tube brought into solution at near boiling point over a flame. Ten to 15 ml. absolute ethanol was added with shaking and a copious crop of crystals appeared. The whole was placed in a refrigerator for some hours and the crystals were then collected in a small Hirsch funnel, washed with absolute ethanol, and allowed to dry in the air.

A yield of 1.30 gm. of practically pure anhydrous ergothioneine was obtained. This is equivalent to 0.26% ergothioneine in the ergot used.

Recrystallization

The product from each batch of blood or ergot was kept separate for assay by colorimetric determination. A specimen of proved purity prepared by Hunter and Eagles (6) served as standard. One of the products gave no diazo test. It proved to be hypoxanthine chloride. The ergot products had small amounts of hypoxanthine present.

The crude products were suspended in water and the insoluble hypoxanthine filtered off.   The filtrates were boiled with a little charcoal and recrystallization carried out as described under the preparation from ergot.

The appearance of the crystals is usually that already pictured by Hunter and Eagles, and described by Eagles and Johnson (2).   Rarely more complete crystal forms are obtained.

The material is practically anhydrous when allowed to dry on the laboratory bench.   For example 99.2 mgm. of air-dried material, after being heated for 1.5 hr. at 110° lost only 0.3 mgm.

The twice recrystallized product melts rather definitely with expansion and decomposition at 280° C. (uncorrected).

No further analysis of our material was deemed necessary.

Discussion

Our failure to obtain good yields of ergothioneine from pig bloods in Edmonton appears to be due to lower ergothioneine content than in pig bloods reported by Hunter (4) in 1928 in Toronto.   The mean ergothioneine value for 12 Toronto pigs was found to be about 26 mgm. per 100 ml. corpuscles. Ten pig bloods tested in Edmonton in August 1948, showed a mean value of about 6 mgm. per 100 ml. whole blood, or less than half of the Toronto value. It should be noted however that the colorimetric determinations in both cases are open to question.

The evidence of Eagles and Vars (3) that diet affects the blood ergothioneine level may be the explanation for our present experience, but an adequate answer awaits further studies based on an accurate method for determining ergothioneine in blood.

In the preparation of ergothioneine from blood Hunter and Eagles (unpublished observation) occasionally observed traces of hypoxanthine in their products.   In our experience hypoxanthine has been more abundant than ergothioneine in some of our crude products. When preparing ergothioneine, free base, it is easy to get rid of hypoxanthine as the free purine is very insoluble in water and readily filtered from neutral solutions of ergothioneine. But if an acid ion is present, as in the case of the preparation of ergothioneine hydrochloride, not only does the purine become soluble but it forms crystals similar to those of ergothioneine, even to showing the bright birefringence colors, under crossed Nicols, that are rather characteristic for ergothioneine and its salts.   The use of cuprous oxide, as has been experienced by Pirie (9) and Lawson and Rimington (8), does not overcome the difficulty.   It is not surprising that cuprous oxide precipitates purines, as its chemical action is likely to be similar to that of the copper sulphate – sodium bisulphite used in the classical method of Krüger and Schmid (7) for purine determinations.

There appears to be little or no purine present in ergot, and nitroprusside reacting SH – groups are, on our own observations, also absent, so that cuprous

oxide is an eminently suitable precipitant for ergothioneine from this source. Our yields from ergot reasonably approach the maximum present in our material.

The use of cuprous oxide is not unattended with some uncertainties. We first applied it to recover ergothioneine from some residues. After the addition of some cuprous oxide suspension the whole solution set to quite a firm gel even at 60° C. The addition of more cuprous oxide liquified the gel and finally a nearly granular precipitate was obtained. On washing with cold 0.5 N sulphuric acid, gelatinous material again appeared, and it was proved that an appreciable amount of material was dissolved. A suitable wash solution was found in 0.5 N sulphuric acid with a little copper present. The solution was prepared by adding a little cuprous oxide to 0.5 N sulphuric acid and bringing to boil for a few minutes. The precipitate was washed with the hot acid. Thorough washing at this stage is essential to remove all ions other than SO_4—subsequently removed by barium hydroxide solution.

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THE DETERMINATION OF ERGOTHIONEINE IN SIMPLE SOLUTION AND IN BLOOD[1]

By G. Hunter

Abstract

The diazo method of Hunter for the determination of ergothioneine in simple solution has been adapted to the Evelyn photoelectric colorimeter. The application of the method to the determination of ergothioneine in blood is described. Suitable blood filtrates are prepared by deproteinization with standard acetic acid oxalate solutions followed by removal of reduced glutathione with Goulard's lead acetate solution, and removal of excess lead with phosphate. By the method precise recovery is obtained of ergothioneine added to human blood.

Introduction

The method described by Hunter (9) for the determination of ergothioneine was regarded as satisfactory for simple solutions, but as of a preliminary nature when applied to blood from various animals. Hunter used reputed tungstic acid filtrates for the determination.

The need for an improved method has recently been emphasized by a number of investigators, as Lawson and Rimington (12) and Astwood and Stanley (1). It was thus decided to restudy the whole problem.

In the interval since the method was first described the advent of the photoelectric colorimeter has necessitated adapting the test in simple solutions to the much more precise modern instruments. In the process of doing this it was found that altering the conditions of the original test in some details considerably increased the sensitivity of the test. Details are given in Part I of this paper for the performance of the test, along with the ergothioneine calibration curve determined for use with the macro attachment of the Evelyn colorimeter.

The problem of elaborating a satisfactory method for blood proved much simpler than might have been anticipated. Part II of this paper describes a method for the determination of ergothioneine in blood, with evidence of its accuracy.

Part I. Determination of Ergothioneine in Simple Solution

EXPERIMENTAL

Reagents

1. The *diazo reagent*, according to Koessler and Hanke (11), is made as follows. In a 50 ml. volumetric flask, immersed in a beaker with water and ice, is placed 1.5 ml. of a solution containing 9 gm. sulphanilic acid and 90 ml. of 37% hydrochloric acid per liter, followed by 1.5 ml. of 5% sodium nitrite

solution.   The mixture is allowed to stand for five minutes, then a further 6 ml. of the nitrite solution is added.   At the end of a further five minutes ice cold water is added to the mark and the contents are mixed.

2. *Alkaline buffer*.   One gm. anhydrous sodium carbonate and 10 gm. anhydrous sodium acetate are dissolved in water and the volume made up to 100 ml.

3. *Ten N sodium hydroxide*, which should be crystal clear and colorless.

Method

A 1 ml. delivery pipette is inserted in the flask carrying the diazo reagent kept immersed in a 1 liter beaker of ice water.   A properly matched Evelyn colorimeter tube is also put into the ice water.

If x ml. is the volume of the test solution to be used $2 - x$ ml. water is added to the tube, followed by 1 ml. diazo reagent and 2 ml. alkaline buffer.   Without delay, x ml. test solution is added and the solutions thoroughly mixed. In the presence of ergothioneine a clear lemon-yellow color rapidly develops. Forty-five seconds are allowed for coupling.   The tube is removed from the beaker and 5 ml. 10 N sodium hydroxide is rapidly run in.   The contents, of the constant volume of 10 ml., are thoroughly mixed.   The tube is held in the hand to warm slightly and facilitate the escape of fine air bubbles produced on the addition of the strong base.   After three to four minutes the solution is free from air bubbles and the final purplish pink color has fully developed. The tube is then read in the colorimeter.

A blank test carried out with 2 ml. of water is used to set the colorimeter at 100% transmittance.   It should be noted that the blank is entirely colorless but has a greater transmittance than distilled water because of its greater refractive index.   If this tube is stoppered it may serve as blank for several weeks.

The color fades very slowly decreasing perhaps 1 galvanometer unit in 15 to 30 min.

CONSTRUCTION OF CALIBRATION CURVE

It was first necessary to choose a suitable filter for the color developed. An absorption curve of the colored solution produced as above was determined in a Coleman spectrophotometer.   The peak of absorption was found at a wave length close to 510 mμ.   The Evelyn filter No. 520, transmitting in the range 495 to 550 mμ., was thus judged to be suitable.   The galvanometer zero was checked.

A solution of ergothioneine containing 0.05 mgm. per ml. was used for the measurements.

The calibration curve is shown in Fig. 1.   It is seen to be linear.   Its mgm./density slope is 0.045.   The concentration of ergothioneine is simply obtainable from the product of slope and density.*

* *The usual calibration constant K_1 in the expression $C = L/K_1$, where C mgm. per ml. is the concentration of the chromogen in the test solution, and L is the photometric density, is 1/10th the reciprocal of the slope, i.e. 1/0.0045 or 222.*
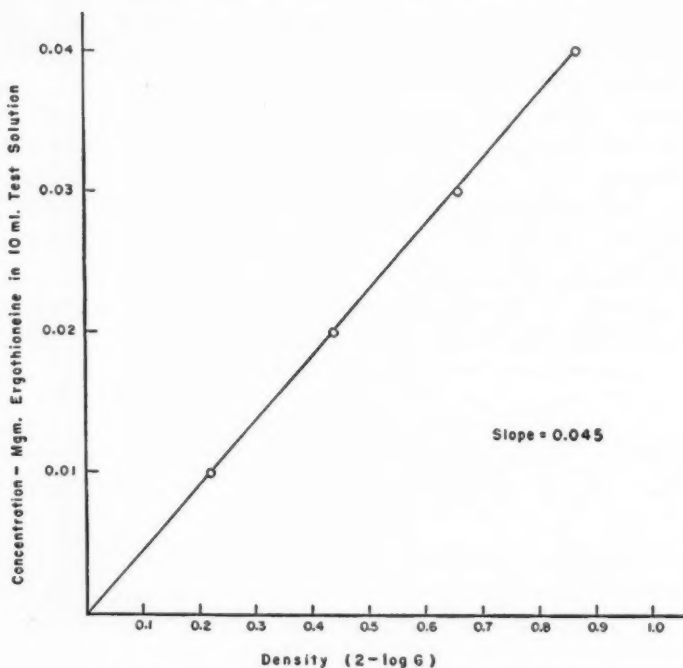
FIG. 1. *Calibration curve for ergothioneine determination.*

Part II. Determination of Ergothioneine in Blood

DISCOVERY OF MAIN FACTORS RELATING TO THE PROBLEM

In resuming the study of the determination of ergothioneine in blood it was unexpectedly found that tungstic acid filtrates rarely gave what could be described as a positive diazo test for ergothioneine. Whether in the course of 20 years the composition of commercial sodium tungstate had changed did not seem at this stage a promising approach so it was decided to remove the proteins from blood simply by heating with a suitable amount of acid, as we found most useful in the preparation procedure.

By treating such filtrates with uranium acetate and removing excess of the latter with sodium dihydrogen phosphate, it was possible to get water clear filtrates giving markedly red colors in the test. But it was rarely that the readings from such filtrates proved to be proportional. Usually, for example, a greater ergothioneine value was calculable when 1 ml. of a 1 : 10 filtrate was used, than when 2 ml. was used. This suggested a substance(s) in the filtrate inhibiting color production, less of it being present in 1 ml. than in 2 ml. of filtrate.

From a stock solution, 0.036 mgm. ergothioneine was placed in each of five test tubes. To four of the tubes was added respectively 2, 1, 0.5, and 0.2 ml.

of a blood filtrate. The volumes in all cases were made to 4 ml. with water. From each tube a 2 ml. portion was taken for the diazo test, and the galvanometer readings were recorded. Table I shows their corresponding density values.

TABLE I

DENSITY VALUES FROM ERGOTHIONEINE SOLUTIONS, WITHOUT AND WITH THE
ADDITION OF DIFFERENT AMOUNTS OF BLOOD FILTRATE.
SEE TEXT FOR DESCRIPTION

Solutions	Density values
Ergothioneine solution	0.398
" " + 2 ml. filtrate	0.257 (poor color)
" " + 1 ml. filtrate	0.284 (poor color)
" " + 0.5 ml. filtrate	0.482 (good color)
" " + 0.2 ml. filtrate	0.444 (good color)

The inhibiting action of 2 and 1 ml. filtrate is clear. But when 0.5 ml. filtrate is used, inhibiting action is at least largely removed, and 0.5 ml. filtrate has contributed (from difference in densities) about 0.004 mgm. to the 0.018 mgm. ergothioneine added. One might thus calculate that this blood has about 16.0 mgm. per 100 ml., whereas on a density value of 0.167, observed on 2 ml. original filtrate, the value is 3.75 mgm. per 100 ml.

This, however, is not a satisfactory solution. Having established the presence of an interfering substance it was desirable to know what it was and, if possible, to find means to remove it.

Reduced glutathione was considered a likely interfering substance. Cysteine was first tested. A few crystals added to the buffered diazo reagent before the addition of ergothioneine completely inhibited coupling. A minute amount of cysteine added permitted some coupling but little red color was produced on the addition of sodium hydroxide. This was very characteristic of the behavior of many blood filtrates treated only with uranium acetate.

On the basis of such observations the blood filtrates were precipitated with a suitable amount of the basic lead acetate preparation known as Goulard's extract (see Part II, Reagents) previously employed by Hunter and Eagles (10) in the preparation of ergothioneine and glutathione from blood. The slight excess of lead was removed from such filtrates by the addition of a suitable amount of a solution of sodium dihydrogen phosphate.

Filtrates thus prepared are suitable for the determination of ergothioneine in all bloods tested, and ergothioneine added to such bloods is quantitatively recoverable. As indicated below this claim is not made for ox blood.

To make the method suitable for routine purposes it was, however, necessary to define more accurately conditions at different points in the procedure. These concern the following main points.

(a) *Deproteinization of Blood*

That blood can be roughly deproteinized by heating with acid is recognized by all, but the method never seems to have been seriously considered in the field of blood analysis.   Were it practicable it has the obvious advantages of yielding a maximum amount of filtrate to which has been added only one simple acid ion.   On the face of it, of course, it would not appear to be workable as a routine method.   Whole bloods differ in composition in a number of directions: hemoglobin content, plasma protein content, and in their proportion of corpuscles to plasma.   The isoelectric points of serum proteins are also distinctly lower than the isoelectric point of hemoglobin.

Despite such considerations the deproteinization of blood by simply heating with acetic acid has been found suitable for our present purposes.   An exhaustive study of the method has not yet been undertaken but precise enough conditions have been determined for the satisfactory removal of proteins from whole blood, corpuscles, and plasma, in dilutions in each case of either 1 : 10 or 1 : 5.   The detailed volumes and standard acid solutions are given below under Part II.

Only in quite exceptional cases do such solutions yield filtrates with more than a trace of color or cloudiness.

(b) *Precipitation with Lead Acetate Solution*

It was sometimes found that when the Goulard's extract was added to the filtrate obtained by heat and acetic acid, that a very small precipitate was obtained and the supernatant liquid often remained cloudy.   Under such circumstances the filtrates are not usually suitable for colorimetry as the glutathione has not been adequately removed.   It was found that with whipped blood and with serum filtrates the lead precipitate did not coagulate satisfactorily.   When oxalate was added to such whipped blood or serum, the subsequent lead precipitation was satisfactory.   It was thus concluded that the precipitation of small amounts of glutathione by lead is an entrainment process.   Hence, to unoxalated blood, oxalate should be added in the usual amount of 1.5 to 2.0 mgm. per ml.   To render the lead precipitation more uniform and certain for routine oxalated bloods, it was decided to incorporate oxalate in the standard acetic acid solutions used for protein precipitation. (See Part II, Reagents.)

(c) *Elimination of Uranium Acetate Precipitation*

Having achieved satisfactory conditions for the removal of protein and the removal of glutathione it appeared that precipitation with uranium acetate might be dispensed with.   This was finally decided on in the light of the observation that its use leads to slight loss of ergothioneine, as shown in the following experiment.

Four lots of 0.5 ml. blood (human) were deproteinized.   The filtrate from one of these, (a), was precipitated directly with lead acetate, but the other three filtrates were precipitated first with the following amounts of a saturated

aqueous solution of uranium acetate:—(b), 0.02 ml.; (c), 0.04 ml.; and (d), 0.06 ml. The ergothioneine found, mgm. per 100 ml., was in each case:—(a), 7.4; (b), 7.0; (c), 6.7; and (d), 6.4.

Whether this loss is due to the direct precipitation of the ergothioneine with the uranium acetate, or to its entanglement in the subsequent lead precipitate has not been determined.

EXPERIMENTAL

Reagents

(a) Acetic Acid – Oxalate Solutions

The solutions as indicated in Table II are used.

TABLE II

ACETIC ACID – OXALATE SOLUTIONS FOR DEPROTEINIZATION OF WHOLE BLOOD, CORPUSCLES, AND PLASMA IN 1 : 10 AND 1 : 5 DILUTIONS

For 1 in 10 dilution	
Whole blood	1 vol. + 9 vol. 0.0045 N acetic acid
Corpuscles	1 vol. + 9 vol. 0.0030 N acetic acid
Plasma	1 vol. + 9 vol. 0.0055 N acetic acid

The above acetic acid solutions are made to contain 160 mgm. sodium oxalate per liter.

For 1 in 5 dilution	
Whole blood	1 vol. + 4 vol. 0.0100 N acetic acid
Corpuscles	1 vol. + 4 vol. 0.0070 N acetic acid
Plasma	1 vol. + 4 vol. 0.0125 N acetic acid

The above acetic acid solutions are made to contain 360 mgm. sodium oxalate per liter.

Of course it is only for special purposes that the plasma solutions are required, as ergothioneine has not been shown to be present in plasma.

(b) Goulard's Extract (8)

Place 220 gm. lead acetate [Pb(CH₃COO)₂ . 3H₂O] and 140 gm. litharge or lead monoxide [PbO] in a 2 liter Erlenmeyer flask with about 1 liter distilled water and bring to boil, with stirring. Maintain at boil for 30 min. Cool and filter through a gravity funnel into a 1 liter volumetric flask. Wash filter with carbon dioxide free water and make to mark. The reagent should be kept stoppered.

(c) Phosphate Solution

Ten gm. sodium dihydrogen phosphate [NaH₂PO₄ . H₂O] to 100 ml. with water.

Method

To 0.5 ml. whole blood in a centrifuge tube add 4.5 ml. 0.0045 N acetic acid – oxalate solution. Mix with a glass rod. Immerse in a faintly boiling

water bath and stir. Coagulation takes place in one to two minutes. By rubbing the glass rod on the side of the tube the gas bubbles are detached from the coagulated protein. Centrifuge. Decant the warm supernatant liquid into another tube and drain well.

Add to the supernatant liquid one drop (0.05 ml.) of Goulard's extract. Mix and tilt the tube to catch any drops on the sides. Centrifuge. Decant as before into another tube, and add one drop phosphate solution. Mix as before and centrifuge. Decant into another tube. This is the test solution – about 3.7 ml. volume.

The test is now carried out with the diazo reagent and buffer as described in Part I for the determination in aqueous solution. At least two aliquots should be taken – usually 2 and 1 ml. – to test proportionality of readings. The amount of ergothioneine in the test solution is obtained as already described.

Recovery of Added Ergothioneine

Ergothioneine added to the original blood is quantitively recoverable by the method, as shown in Table III.

TABLE III

RECOVERY OF ERGOTHIONEINE ADDED TO HUMAN BLOODS

Subject	Mgm. ergothioneine per 100 ml.			
	Original blood	Added	Found	Calculated
M	4.15	2.85	7.25	7.00
T	(a) 1.25	2.30	3.50	3.55
	(b) 1.25	4.60	6.00	5.85
S	(a) 4.35	4.25	8.60	8.60
	(b) 4.35	8.50	12.90	12.85

The recovery of ergothioneine, added to the original blood so that it goes through the whole extractión process in the preparation of the final filtrate for the test is perhaps the best criterion of the method. Subject T was a case of hyperthyroidism and shows the lowest ergothioneine value we have so far found in blood. For the reason that the original ergothioneine content was very low this blood presents a rather rigorous criterion for the recovery of small amounts of ergothioneine added to it.

Distribution of Ergothioneine in Blood

Table IV serves as a protocol of the colorimetric assay method and as a further check of its accuracy. The concordance of values found for whole blood with those calculated from corpuscles and plasma values would indeed have been better had the plasma also been precipitated at a 1 : 10 dilution.

TABLE IV

PROTOCOL FOR TWO HUMAN BLOODS

Subject	Cor-puscles, vol. %	Analysis material	Filtrate dilution	Aliquot for test, ml.	Photo-metric density	Ergo-thioneine in 0. 1 ml. blood, mgm.	Ergothioneine mgm./100 ml.	
							Found	Cal-culated*
K	48.05	Whole blood	1 : 10	2	0.235			
				1	0.121	0.0054	5.4	5.5
		Corpuscles	1 : 10	2	0.475			
				1	0.233	0.0105	10.5	
		Plasma	1 : 5.5	2	0.069			
				1	0.036	0.0009	0.9**	
S	33.33	Whole blood	1 : 10	2	0.258			
				1	0.126	0.0057	5.7	6.1
		Corpuscles	1 : 10	2	0.688			
				1	0.347	0.0156	15.6	
		Plasma	1 : 5.5	2	0.109			
				1	0.055	0.0013	1.3**	

* *From content of corpuscles and plasma and volume % corpuscles.*
** *Not to be interpreted as ergothioneine, see text.*

It was a surprise to find that a significant yellow color is always found in plasmas even with a minimum of corpuscles or their constituents present. Expressed as ergothioneine this color is usually equivalent to 0.5 to 1.0 mgm. per 100 ml. plasma.   There is no reason to believe that any of this color is due to ergothioneine.   It is most likely due to histidine and (or) tyrosine. If we assume that 1 mgm. of each of these amino acids is circulating in 100 ml. plasma then, at a 1 : 5 dilution with a 2 or even 1 ml. aliquot they come just within the range of sensitivity of the diazo reagents.   However with the limitation of the coupling time to 45 sec. perhaps a negligible proportion of the color arises from histidine, and the greater amount from tyrosine, which couples faster than histidine.

The interference indeed is considered to be insignificant in the determination of ergothioneine in whole blood or corpuscles, but it is of interest in the still open question as to whether plasma has a low level of circulating ergothioneine. Values found for plasma by the present method should not be interpreted as indicative of the presence of ergothioneine.

It may be observed from Table IV that the 1 ml. aliquot of filtrate gives very nearly in all cases just half the ergothioneine value obtained from the 2 ml. aliquot.   This is so, perhaps fortuituously, also in the case of the plasmas. In the occasional blood filtrate, a proportionately lower value is found with the 2 ml. aliquot in which case the color hue is not likely to be of a characteristic bright purplish pink but more yellow, indicating the presence of interfering

substance (s).   In most of such cases the use of a 0.5 ml. aliquot will give a color proportional to that of the 1.0 ml. aliquot in which cases the values for the 2 ml. aliquot should be discarded.

To date, upwards of 100 whole bloods have been tested with only occasional evidence of interference with color development.   From several ox bloods examined there is evidence of gross interference by some constituent not yet identified.   Combined uric acid, already demonstrated to be present in ox blood by Bulmer, Eagles, and Hunter (6), and earlier by Davis, Newton, and Benedict (7), may be a factor in the present problem.

The simplicity of the present method may here be emphasized.   Two to four test filtrates can be prepared in 10 to 15 min., with simultaneous depro-teinization and the use of a modern clinical centrifuge.   It should be relatively simple to adapt the method to the micro Evelyn attachment.

Discussion

In a note on the determination of ergothioneine in blood filtrates Behre (2) in 1932 stated that "no great reliance can be placed on figures so far obtained." It is unlikely that such a statement repeated today could be contradicted.

The observation of Benedict and Newton (5) that glutathione is precipitated by silver lactate, used for the determination of uric acid in blood, would appear to discredit all ergothioneine values reported on the basis of the use of uric acid reagents.   It is thus unnecessary here to discuss the various publications on the ergothioneine content of blood obtained without adequate fractionation by such unspecific reagents.

There is one point, however, of some interest in the history of this subject. It has been stated at the beginning of this paper that tungstic acid filtrates of blood generally failed to give positive diazo tests for ergothioneine whilst the values reported in 1928 by Hunter (9) were obtained from reputed tungstic acid filtrates.

Benedict and Newton (3) recommended the use of molybdic acid filtrates on evidence that ergothioneine is precipitated by tungstic acid.   On their further evidence that molybdic acid precipitates some glutathione, they (4) later recommended a tungstic-molybdic acid mixture, and stated that the ergothioneine values in such filtrates are about twice those obtained in tungstic acid filtrates.

A pig blood known to contain 14.8 mgm. ergothioneine per 100 ml. was precipitated with tungstic acid at a 1 : 10 dilution in the usual way, and a portion similarly treated with molybdic acid according to Benedict and Newton (3).   Diazo tests were carried out on a 2 and 1 ml. aliquot of each, and remarkably enough, good ergothioneine diazo colors were obtained from the molybdic acid filtrate but poor yellow colors on the tungstic acid filtrate. The 2 ml. aliquot of the molybdic filtrate indicated 9.0 mgm. and the 1 ml.

filtrate 10 mgm. ergothioneine per 100 ml. or about 60% of the true ergo-thioneine content. Significantly both filtrates showed a positive nitro-prusside test indicative of reduced glutathione.

This observation would appear to solve the puzzling discrepancy between the observations of Hunter in 1928 and the present ones. It is highly probable that the commercial tungstic acid used by Hunter in 1928 was composed partly of molybdic acid.

No attempt need here be made to explain the different behaviors of tungstic and molybdic acid, as the present method is not concerned with either.

It may, however, be stated that the values already published by Hunter (9) on the ergothioneine content of bloods probably represent not more than 75% and in some cases much less of the true values for the bloods reported.

It is hoped to report further, at an early date, on the normal levels of ergo-thioneine in human and animal bloods.

It would appear that the method here described for the determination of ergothioneine in blood will make possible a better understanding of its physio-logical and perhaps clinical significance.

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NOTE ON THE ERGOTHIONEINE CONTENT OF ERGOT FROM DIFFERENT PLANT HOSTS[1]

By G. Hunter, S. G. Fushtey, and D. W. Gee

Abstract

A method is described for the determination of ergothioneine in ergot sclerotia obtained from the host plants *Agropyron*, *Bromus*, *Calamagrostis*, *Elymus*, *Hordeum*, and *Secale*.

Ergothioneine is present in all ergots examined at levels from 0.157 to 0.531%. The average concentration in ergot of barley is 0.376% and in ergot of rye 0.336%.

Introduction

As ergothioneine can not yet be obtained by synthesis and as large volumes of blood, under the most favorable conditions, must be processed to obtain the substance in amounts useful for physiological or clinical studies, it was decided to examine more closely the ergothioneine content of ergot.

The only indications of the percentage of ergothioneine in ergot are to be found in the yields obtained from the few records of its isolation available in the literature. In chronological order such are: Tanret (6) 0.10% as hydrated hydrochloride; Eagles (1) 0.065% as anhydrous free base; Pirie (5) 0.18% as anhydrous free base, and Hunter, Molnar, and Wight (4) 0.26% as anhydrous free base.

It appeared that the method of Hunter (3) might, without much difficulty, be applied to the determination of ergothioneine in ergot sclerotia collected by one of us (S.G.F.) in the course of another study. This paper describes a method for the determination of ergothioneine in ergot and the values found in the fungus picked from different host plants.

Experimental

All analyses were carried out on air-dried material and the values found expressed on the same basis.

The routine analytical procedure aimed at a sample weighing 30 to 50 mgm. of material. The weight of many single sclerotia fell in this range. Large sclerotia were halved and, when the sclerotia were very small, as from *Calamagrostis canadensis*, 20 to 30 sclerotia were required for the sample.

Method

Thirty to 50 mgm. of material is placed in a 15 ml. graduated centrifuge tube. About 4 ml. water and 1 drop of 2 N acetic acid are added, and the tube is placed in a boiling water bath for 10 min. The contents are then poured into a small mortar, the tube being carefully set aside for return of this material.

The ergot is finely ground, and quantitatively returned to the tube by washing pestle and mortar with a further 5 to 6 ml. water. The tube is placed in the boiling water bath for a further 5 min. It is centrifuged. The total volume, here recorded, is taken for the dilution factor in the calculation. The supernatant liquid is carefully transferred into a plain 16 ml. test tube fitting the centrifuge and treated with a saturated solution of uranium acetate to slight excess as determined by test with a 5% solution of potassium ferrocyanide on a tile with a small drop of the supernatant fluid. From 0.1 to 0.2 ml. of the uranium solution is usually required. The tube is again centrifuged, and the supernatant fluid transferred to another 16 ml. test tube. The slight excess of uranium is removed from this solution by the addition of one drop (0.05 ml.) of 10% solution of sodium dihydrogen phosphate. The solution should be well mixed and allowed 15 to 20 min. for complete coagulation of the precipitate before centrifugation. The supernatant liquid is poured into another tube. This is the test solution.

Colorimetric Determination

Aliquots from the test solution are measured by the diazo method described by Hunter (3). At least two different aliquots should show strict linear transmittance.

The values for the ergots examined are shown in Table I.

Discussion

(a) Of Method

It may be seen that the preparation of the ergot extract suitable for colorimetric determination follows the first two steps in the isolation procedure used by Hunter, Molnar, and Wight (4). In the isolation procedure however the ergot was extracted with only about five volumes of water while here about 200 volumes of water are used. The relatively high concentration of ergothioneine in ergot permits this large dilution at the outset and makes negligible the volume occupied by the ergot residue. When the material is finely ground as described it is assumed that after the second heating the ergothioneine is evenly distributed in solid and liquid.

From this point on, small amounts of ergothioneine are of course left behind in each transfer and the approximately 10 ml. volume recorded is diluted by about 2% by the subsequent addition of the uranium and phosphate solutions, so that the values finally recorded for ergothioneine in the ergot are below the real values by probably 3 to 5%.

At the dilution used for extraction, aliquots greater than 1 ml. tend to give values less than those obtained by smaller aliquots—indicating the presence of traces of substance(s) in some test solutions interfering with color development. By the use of smaller aliquots such substances are diluted beyond their range of interference and strictly linear transmittances are nearly always easily obtainable. For this reason the method of determination was considered adequate for present purposes.

(b) On Occurrence in Ergot

Except in a few cases the analyses were carried out on single sclerotia in triplicate. It may be seen from Table I that about a 50% difference in

TABLE I

| Host	% Ergothioneine	
	In separate samples	Average
Agropyron Smithii Rydb.	0.180 0.261	0.220
Agropyron repens (L.) Beauv.	0.333 0.430 0.305	0.356
Bromus inermis Leyss	0.381 0.362 0.530	0.424
Calamagrostis canadensis (Michx.) Beauv.	0.162 0.157	0.160
Elymus innovatus Beal	0.310 0.329	0.320
Hordeum vulgare var. *trifurcatum* Schlecht	0.334 0.318 0.385	0.344
Hordeum vulgare L. (immature, 1948)	0.483 0.531 0.442	0.485
Hordeum vulgare L. (mature, 1948)	0.325 0.455 0.372	0.384
Hordeum vulgare L. (mature, 1946)	0.402 0.398	0.400
Hordeum vulgare L. (mature, 1947)	0.231 0.298	0.265
Secale cereale L.—1948—Drumheller	0.299 0.183 0.215	0.232
Secale cereale L.—1948—Edmonton (mature)	0.383 0.326 0.368	0.359
Secale cereale L.—1948—Edmonton (immature)	0.233 0.293 0.202	0.243
Secale cereale L.—1947—Edmonton (mature)	0.448 0.406 0.401	0.418
	0.364 0.474 0.445	0.428

percentage ergothioneine may be found in different samples of the same variety of ergot, though generally in our few observations the variation is much less. The five specimens of ergot of barley recorded in Table I show an over-all average of 376 mgm. ergothioneine per 100 gm. with a range from 531 to 231. In the case of the five specimens of ergot of rye the over-all average is 336 mgm. ergothioneine per 100 gm. with a range from 474 to 183. The ergots from *Bromus* and *Agropyron* contain ergothioneine in about the same concentrations as ergots from barley and rye. The lowest values were found in the ergot from *Calamagrostis* but here the sclerotia are very small (little more than 1 mgm. each) and some extraneous material may have been in the sample.

The biochemical synthesis of the thiolglyoxaline nucleus in the only two sources in which it is yet known to exist namely, the fungus ergot, and mammalian red blood corpuscles, is quite obscure. The only suggestion so far offered is to be found in the paper by Eagles and Vars (2) who give some evidence that a thiolglyoxaline nucleus is contained in zein, the main protein present in maize. The findings of Eagles and Vars have not been confirmed so the presence of the thiolglyoxaline nucleus in any protein has yet to be proved.

Assuming, for a moment, the correctness of Eagles and Var's observation that a thiolglyoxaline exists in zein, an explanation of their findings with pigs fed maize and pigs fed garbage is possible. Yet it is difficult to believe that such an explanation is adequate for the general occurrence of ergothioneine in blood corpuscles. The cereal proteins have not as yet been examined for the thiolglyoxaline nucleus. Were it found to be present in the proteins of rye, or barley or other ergot hosts perhaps there would be no problem of special interest for the plant or animal physiologist. But as matters now stand the origin of the thiolglyoxaline nucleus in red blood corpuscles and in ergot would appear to be a subject of considerable biochemical interest.

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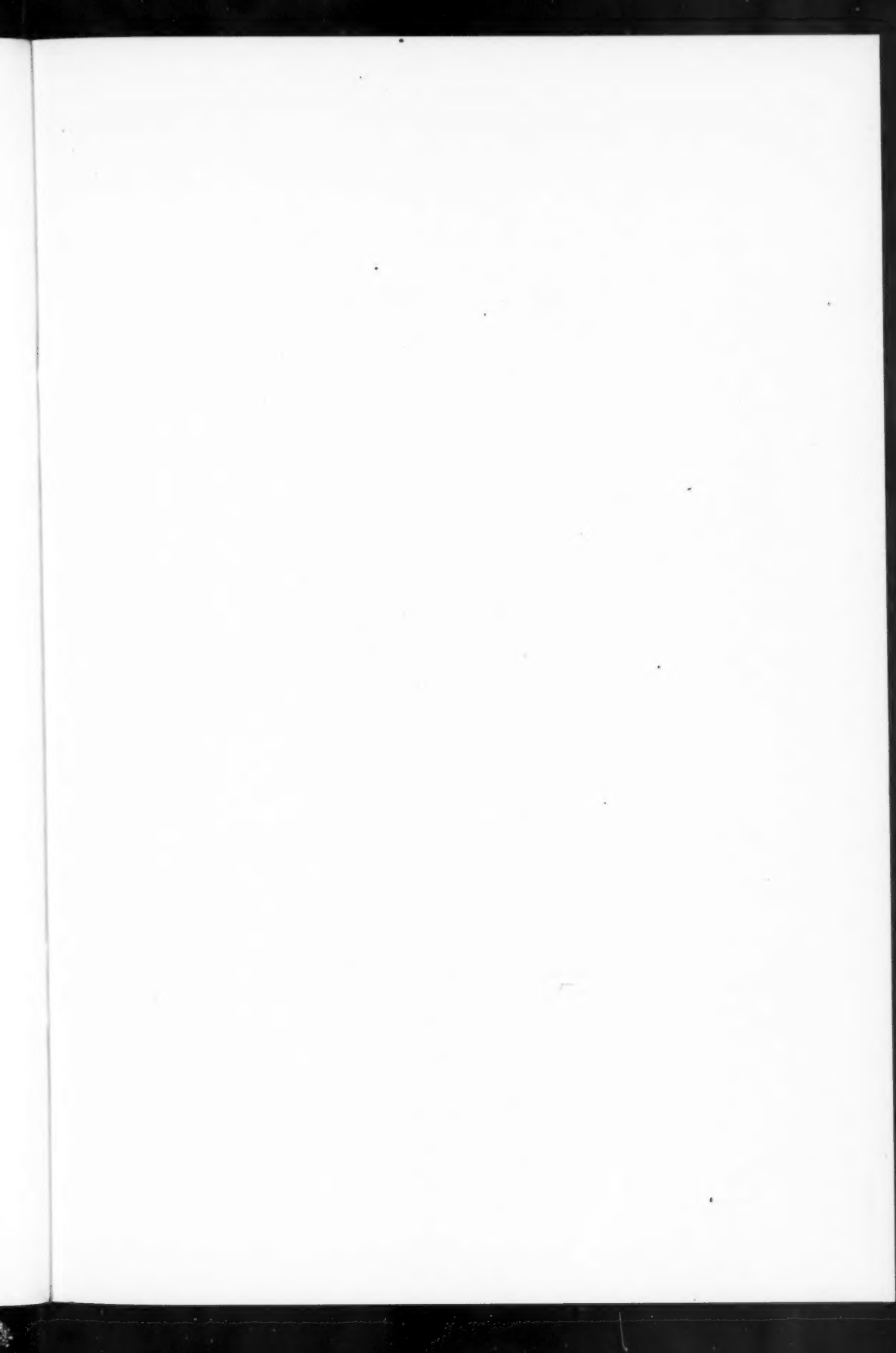
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